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Diet-induced changes in Ucp1 expression in bovine adipose tissues

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Abstract

Brown adipocytes, which regulate non-shivering thermogenesis, have been believed to exist in a limited number of mammalian species, and only under limited physiological conditions. Recent discoveries indicate that adult humans possess a significant number of functional brown adipocytes. This study explores the regulatory emergence of brown adipocytes in white adipose tissue (WAT) depots of fattening cattle. RT-PCR analyses indicated significant expression of *Ucp1*, a brown adipocyte-specific gene, in the WAT of 31-month-old Japanese Black steers. Immunohistochemical analysis revealed that *Ucp1*-positive small adipocytes were dispersed in the subcutaneous WAT. Next, we examined expression level of *Ucp1* and other brown adipocyte-selective genes such as *Pgc1α, Cidea, Dio2, Cox1, Cox7a1* and *Cox8b* in WAT of 30-month-old steers fed either diet with low protein/energy content (roughage diet) or that with high protein/energy content (concentrate diet) for 20 months. *Ucp1* expression in the subcutaneous WAT was significantly higher in the concentrate diet group than in the roughage diet group. Furthermore, the higher *Ucp1* expression levels were limited to the subcutaneous WAT, and no differences between groups were detected in the mesenteric, perirenal, intermuscular or intramuscular WAT. Expression of *Dio2*, *Cox1* and *Cox8b* was higher in the subcutaneous WAT but not in the mesenteric WAT of the concentrate diet group. Furthermore, expression of *Prdm16*, a positive regulator of differentiation toward brown adipocyte-lineage cells, and expression of *leptin*, a molecule that enhances activity of brown adipocytes, were significantly higher in the subcutaneous WAT of the concentrate diet group. This study demonstrates the presence of brown adipocytes in WAT depots of fattening cattle, and suggests the diet-related modulation of expression of genes predominantly expressed in brown adipocytes.

1. Introduction

There are two major types of adipose tissues in mammals: white and brown. White adipose tissue (WAT), which is dispersed throughout the body in mammals [13], is specialized for the storage of excess energy. WAT contains all of the enzymatic machinery necessary to produce triacylglycerols from fatty acids, either those synthesized *de novo* or imported from circulating lipoproteins. In addition, WAT plays a central role in the regulation of energy balance by acting as the site of the synthesis and secretion of molecules called adipokines, including leptin [11, 30]. In contrast, brown adipose tissue (BAT) is specialized to dissipate chemical energy in the form of heat in response to cold or excess feeding [1, 14, 22, 23, 38]. The thermogenic function of BAT results from the expression of a series of genes related to high mitochondrial content, as well as elevated cellular respiration largely uncoupled from ATP synthesis. The uncoupling occurs through expression of uncoupling protein 1 (Ucp1), a brown adipocyte-specific mitochondrial protein that promotes proton leak across the inner mitochondrial membrane [4, 13].

Thermogenesis in brown adipocytes has hitherto been considered to occur in a limited number of animal species, including small rodents, and under the limited (patho)physiological status such as newborn humans and patients with phenochromocytoma [4, 13]. Recently, a significant amount of a functional brown adipocyte depot was identified in adult humans via integrated positron emission tomography-computed tomography (PET-CT) studies using an ^{18}F-labeled glucose analogue, fluorodeoxyglucose, as a tracer, and by the immunohistochemical analyses to detect Ucp1 [9, 31, 45, 47]. The finding that the sizes of the BAT depots are inversely correlated with body mass index [9, 31, 45, 47] has fuelled considerable interest in the therapeutic potential of brown adipocytes in obesity and obesity-related diseases [44].

Ucp1-positive brown adipocytes are also found interspersed in the WAT of rodents and adult humans, which are therefore called “brown in white” (brite) adipocytes, inducible brown fat cells, or beige cells [5, 18, 32]. Furthermore, *Ucp1* mRNA was detected in the subcutaneous WAT of adult humans [29, 42, 49]. In view of the large amount of WAT depots, the brite adipocytes may be the major brown adipocyte throughout the human body [42].

Beef cattle are raised as industrial animals, and fattening efficiency is one of the determining factors of the economy of beef production. Unlike the case of adult humans, the emergence of brite adipocytes, which dissipate energy as heat, is not preferable in beef cattle. Thus, determining the factors affecting the induction and activity of brite adipocytes is important in beef cattle and humans alike, although the presence of brite adipocytes has not yet been established in fattening cattle. While *Ucp1* expression was detected in the subcutaneous WAT of fetal calves [20, 37], the expression level was decreased to the detection limit at birth. Expression of *Ucp1* could not be detected in the subcutaneous, perirenal and intermuscular WAT of mature cattle [20, 25, 26, 39, 40]. However, in previous studies, *Ucp1* expression was examined by Northern blot or slot blot analyses, techniques that are generally insensitive methods compared with quantitative real-time RT-PCR analyses. Therefore, it is possible that the *Ucp1* expression in WAT of fattening cattle was previously overlooked.

The objectives of the present study are to clarify the expression of Ucp1 in WAT depots of fattening cattle, and effects of diets on *Ucp1* expression. Our findings demonstrate that *Ucp1* is expressed in the WAT of fattening cattle and that the expression level in the subcutaneous WAT is increased by consuming a diet with higher protein and energy density. In view of the substantial parallel increase in expression of *Ucp1* and that of the

other brown adipocyte-selective genes, we propose the diet-related emergence of brite adipocytes in fattening cattle.

2. Materials and methods

2.1. Animals and feeds

In Experiment 1, four Japanese Black steers aged 21 months were fed roughage (rice straw) with 38% total digestible nutrients (TDN) and 5% crude protein (CP), and a concentrate mixture consisting of barley, corn, wheat bran, rice bran and soybean meal with 74% TDN and 12% CP on an ad libitum basis for 10 months. The steers were raised in a stall covered with sawdust. They were allowed free access to drinking water and a mineral block (Cowstone, Nihon Zenyaku Kogyo, Koriyama, Japan). The perirenal and subcutaneous WAT depots were collected from the steers aged 31 months in a commercial slaughterhouse, where steers were exsanguinated after stunning. Tissue samples were promptly collected. The perirenal WAT depot samples were frozen in dry ice and stored at -80°C until samples underwent total RNA extraction. The subcutaneous WAT depot was washed with phosphate-buffered saline (PBS) and fixed for immunohistochemical analysis.

In Experiment 2, 8 Japanese Black steers were used. Experiment 2 was the same experiment reported by Yamada and Nakanishi [48]; data on daily feed intake and body composition were previously shown [48]. The steers were raised in a stall covered with sawdust. They were allowed free access to drinking water and a mineral block (Cowstone, Nihon Zenyaku Kogyo, Koriyama, Japan). Feeds were individually provided by door feeding system (Orion Machinery, Kyoto, Japan). The steers were fed roughage (orchardgrass hay) with 56% TDN and 8% CP and a concentrate mixture

consisting of corn, barley, wheat bran, rice bran and soybean meal (Nasuno for Wagyu Fattening; JA Higashi-nihon Kumiai Shiryou, Ota, Gunma, Japan) with 88% TDN and 15% CP as described below. At 10 months of age, the steers were allotted by body weight to one of two groups: the roughage diet group (n = 4) or the concentrate diet group (n = 4). The roughage diet consisted of 35% roughage and 65% concentrate mixture, whereas the concentrate diet contained 10% roughage and 90% concentrate mixture on a TDN basis. The roughage was given on an ad libitum basis to steers in the roughage diet group. The amount of concentrate in the roughage diet group was determined as follows: the amount of TDN required for achievement of body weight gain of 0.7 kg/day was obtained from the Japanese Feeding Standard for Beef Cattle [27], and the concentrate that corresponded to 65% of the required TDN was provided to the steers. To eliminate the influence of the total TDN intake between groups, the steers were pair-fed for 20 months; weekly TDN intake was measured in the roughage diet group, and amount of feed with average TDN content in the former week in the roughage diet group was provided to steers in the concentrate diet group. At 30 months of age, steers were slaughtered in a commercial slaughterhouse, and adipose samples from five types of WAT depots (subcutaneous, mesenteric, perirenal, intermuscular, and intramuscular) were collected [48]. All animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals (National Institute of Livestock and Grassland Science).

2.2. Immunohistochemistry

Rabbit polyclonal antibody to human Ucp1 (ab10983) was obtained from Abcam (Cambridge, MA) to examine immunolocalization in the subcutaneous WAT. According to the manufacturer, this antibody is predicted to recognize bovine Ucp1. After fixation with Bouin’s solution for 24 h at room temperature, the samples were

dehydrated, embedded in paraffin, and sectioned to 4 μm thickness. Endogenous peroxidase was blocked using 0.3% H_2O_2 in methanol for 20 min at room temperature. The sections were washed with PBS, and treated using blocking solution (Histofine SAB-PO(R); Nichirei Biosciences, Tokyo, Japan) for 10 min at room temperature. After washing with PBS, the sections were incubated with the anti-Ucp1 antibody (diluted 1:400) overnight at 4°C. The sections were then washed with PBS and incubated with a biotinylated goat anti-rabbit secondary antibody (Histofine SAB-PO(R)) for 10 min at room temperature. After washing with PBS, the sections were incubated with peroxidase-conjugated streptavidin for 5 min at room temperature. After washing with PBS, the DAB substrate kit (Nichirei Biosciences) was applied to the sections for 2 min at room temperature, followed by counterstaining with hematoxylin. The sections were then dehydrated and mounted. The experiments were repeated at least three times, and the positive staining was reproducibly detected.

2.3. RNA isolation, conventional RT-PCR and quantitative RT-PCR

Total RNA isolation and cDNA synthesis were conducted as described by Yamada and Nakanishi [48]. The cDNA, reverse-transcribed from 10 ng of total RNA, was used as a template for conventional RT-PCR or quantitative RT-PCR (qRT-PCR). The oligonucleotide primers for conventional RT-PCR and qRT-PCR are presented in Table 1. Conventional PCR was performed in a total volume of 10 μl containing 1× *Ex-Taq* buffer with 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer, and 1.25 U of an *Ex-Taq* HS DNA polymerase (TaKaRa, Kyoto, Japan). The PCR profile of conventional RT-PCR is as follows: after denature for 10 sec at 95°C, 35 cycles consisting of 5 sec at 95°C and 20 sec at 60°C. The PCR products were separated in a 2% agarose gel in 1× TAE and visualized with ethidium bromide. In Experiment 2, the qRT-PCR was performed as described previously [12]. The C_t value was determined,

and the abundance of gene transcripts was calculated from the C_t value by normalizing against *Hprt1*; *Hprt1* expression is frequently used to evaluate expression level of gene of interest as a reference [28]. Expression of *Ucp1*, *Prdm16* and *leptin* was examined in 5 WAT depots, whereas that of *Pgc1α*, *Cidea*, *Dio2*, *Cox1*, *Cox7a1* and *Cox8b* was done in the subcutaneous WAT and the mesenteric WAT. The expression in the roughage diet group in each WAT depot was set to 1, and the expression in the concentrate diet group was expressed as the value relative to that in the roughage diet group.

2.4. Statistical analyses

Data are expressed as the mean ± SEM. Data were log-transformed to provide an approximation of a normal distribution before analysis. Differences between the dietary groups were examined using unpaired *t*-test. Differences of $P < 0.05$ were considered significant. A tendency to a difference was considered to be present when $0.05 \leq P < 0.10$.

3. Results

3.1. Expression of Ucp1 in WAT of fattening cattle

Experiment 1 was performed as the first step to explore the presence of brite adipocytes in fattening cattle. Conventional RT-PCR was conducted to examine *Ucp1* expression using total RNA prepared from the perirenal WAT depot of 31-month-old cattle (Fig. 1). A band with the expected size (235 bp) was reproducibly detected only in the sample treated with reverse transcriptase.

A representative result on immunohistochemical analyses to detect Ucp1 expressing cells is shown in Fig. 2. Ucp1-positive small adipocytes were scatteredly located

between white adipocytes in the subcutaneous WAT of 31-month-old cattle (Fig. 2).

3.2. Regulatory expression of Ucp1 in subcutaneous WAT related to diet

In Experiment 2, because the steers were pair-fed, intake of TDN and CP was comparable between the dietary groups [48]. In addition, body weight and daily weight gain were not different between the groups [48]. Furthermore, there were no differences in the weights of carcass bone, lean, and adipose tissue between the groups [48].

We evaluated diet-related changes in expression of *Ucp1* in WAT of fattening cattle. Expression of *Ucp1* in the subcutaneous WAT was significantly higher in the concentrate diet group than in the roughage diet group ($P = 0.01$, Fig. 3). In contrast, the expression in the other regions of WAT depots was not significantly different between the groups, although it tended to be higher in the concentrate diet group in the intramuscular WAT ($P = 0.09$).

Furthermore, gene expression levels of *Pgc1α*, *Cidea*, *Dio2*, *Cox1*, *Cox7a1* and *Cox8b* were examined; these genes are involved in up-regulating *Ucp1* expression and in activating brown adipocytes [35]. Consistent with increased expression of *Ucp1*, expression levels of the brown adipocyte-selective genes in the subcutaneous WAT were generally higher in the concentrate diet group than in the roughage diet group. Specifically, expression levels of *Dio2* ($P = 0.03$), *Cox1* ($P = 0.007$) and *Cox8b* ($P = 0.009$) were significantly higher in the concentrate diet group (Fig. 4). However, expression levels of the brown adipocyte-selective genes were comparable between the groups in the mesenteric WAT.

Prdm16 and leptin are molecules involved in the differentiation and activation of brown

adipocytes, respectively [7, 33]. Dietary effects on the expression of *Prdm16* and *leptin* were similar to those on *Ucp1* expression, as higher expression in the concentrate diet group was detected in the subcutaneous WAT depots (P = 0.006 and P = 0.003, respectively, Fig. 5). In addition, *leptin* expression in the intramuscular WAT depot was higher in the concentrate diet group than in the roughage diet group (P = 0.02, Fig. 5B).

4. Discussion

This study clarifies that 1) brite adipocytes are present in WAT depots of fattening cattle, 2) expression levels of *Ucp1* is increased in response to feeding the concentrate diet, which is limited in the subcutaneous WAT, and 3) expression levels of brown adipocyte-selective genes are also increased in the subcutaneous WAT but not in the mesenteric WAT of steers fed the concentrate diet. In adult humans, the expression level of *Ucp1* in WAT depots is affected by body mass index and insulin sensitivity, implicating that brite cell activity is modulated by metabolism [42]. The emergence of brite adipocytes is also affected by environmental and pharmacological factors [2, 8, 10], as cold acclimation or CL316,243, a β_3-adrenergic receptor agonist [50], induced brown adipocytes in WAT [2, 17, 46]. Furthermore, brown adipocytes in WAT depots are also induced in genetically engineered mice, e.g., mice forced expression of Prdm16 in adipocytes [34]. This study indicates that diet can modulate expression of *Ucp1* and brown adipocyte-selective genes, suggesting further physiological control over the emergence or activation of brite adipocytes.

The diet-related changes in expression levels of *Pgc1α*, *Cidea*, *Dio2*, *Cox1*, *Cox7a1* and *Cox8b* in the subcutaneous WAT were basically similar to those of subcutaneous *Ucp1* (Fig. 4); the expression levels in the concentrate diet group were generally higher than

256 those in the roughage diet group. Especially, the higher expression of *Dio2*, *Cox1* and
257 *Cox8b* was statistically significant. All these gene products are involved in function of
258 brown adipocytes as heat producing cells; in murine BAT, expression of Dio2, an
259 enzyme catalyzing the reaction of thyroxine to bioactive triiodothyronine [3], is
260 increased during cold exposure [36], and triiodothyronine stimulates thermogenesis in
261 BAT through activation of sympathetic nervous system [21]. In addition, expression of
262 Cox1, a cyclooxygenase, is up-regulated in the subcutaneous WAT depot in mice
263 during cold exposure, and the increased expression was required for up-regulation of
264 *Ucp1* expression and heat production [24]. Furthermore, consistent with the fact that
265 brown adipocytes are rich in mitochondria [4], expression of *Cox8b*, a component of
266 mitochondrial cytochrome c oxidase, is higher in BAT depot than in WAT depot [35].
267 Taken higher *Ucp1* expression in the subcutaneous WAT of steers fed the concentrate
268 diet with the results together, number of functional brown adipocytes in the
269 subcutaneous WAT possibly increased in response to feeding the concentrate diet.
270
271 The present study revealed that the diet-related modulation of *Ucp1*, *Dio2*, *Cox1* and
272 *Cox8b* expression was detected in the subcutaneous WAT but not in the mesenteric
273 WAT. The cold-induced emergence of Ucp1-positive adipocytes was detected in the
274 inguinal WAT (a representative subcutaneous WAT), but not in the parametrial WAT (a
275 representative visceral WAT) in mice [2]. Treatment with Fgf21 induced *Ucp1*
276 expression in the inguinal WAT but not in the epididymal WAT, another representative
277 visceral WAT [10]. Thus, the more prominent regulation of brite adipocyte induction in
278 the subcutaneous WAT than in the visceral WAT may be a common feature in
279 mammals.
280
281 Prdm16 and leptin are possibly involved in regulating the emergence of brite adipocytes.

Expression of *Prdm16* in the subcutaneous WAT was higher in the concentrate diet group than in the roughage diet group, which coincided with the dietary effect on expression of *Ucp1* and brown adipocyte-selective genes. Prdm16 is a transcriptional co-regulator that stimulates the development of brown adipocytes [19, 33, 34]. Treatment with leptin stimulated energy expenditure [16] and expression of Ucp1 in BAT as well as WAT depots [7] in leptin-deficient ob/ob mice. Leptin-induced up-regulation of the *Ucp1* expression was reduced in melanocortin receptor 4-deficient mice [51]. Furthermore, leptin increased the production of α-melanocyte-stimulating hormone in the hypothalamus, indicating the involvement of the leptin-melanocortin pathway in activating brown adipocytes [15]. Thus, at least two reasons are possible for the concentrate diet-induced Ucp1 expression in the subcutaneous WAT: 1) brown adipocyte differentiation is locally stimulated through up-regulation of Prdm16 expression, and 2) enhanced expression of leptin, which is produced in and secreted from white adipocytes [43], systemically acts as a stimulator of brown adipocyte activation.

Dispersed brite adipocytes in large subcutaneous WAT masses are suggested to cumulatively represent substantial brown adipocyte activity in adult humans, although they are not detected by the integrated PET-CT method [42]. Expression levels of *Ucp1* in the subcutaneous WAT were significantly affected by diet, but body weight gain and body composition in cattle used in this study were comparable between the groups [48]. We postulate that the concentrate diet-induced activation of brite adipocytes is masked by the enhanced anabolic activity in cattle. In support of this, plasma concentrations of insulin and Igf-1, both with potent anabolic effects [6], linearly increased with concentrate proportions in cattle feed under the condition of equal energy ingestion between groups, suggesting an intrinsic anabolic effect of the concentrate diet [41].

Thus, suppressing the induction of brite adipocytes in fattening cattle maintained on the concentrate diet may improve the fattening efficiency significantly.

Here we show the presence of brown adipocytes in WAT depots of fattening cattle based upon the expression of brown adipocyte-selective genes, and the regulatory expression of the genes in response to diet. The present results point out a novel possibility on the concentrate diet-specific emergence or activation of the subcutaneous brite adipocytes. To uncover the precise role of brown adipocytes in WAT depots in relation to the diet, further studies are needed to clarify 1) the molecular bases of the concentrate diet-induced up-regulation of *Prdm16* and *leptin* expression, 2) a detailed explanation as to why the diet-induced modulation of *Ucp1* and *Prdm16* expression occurs in the subcutaneous WAT but not in the visceral WAT, and 3) the physiological significance of the induced brown adipocytes.

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Figure legends

Fig. 1. Expression of Ucp1 in perirenal WAT depot of fattening cattle

Total RNA was isolated from the perirenal WAT depot of cattle aged 31 months, and cDNA was prepared by treatment with (RT+) or without (RT-) reverse transcriptase. PCR was performed to detect *Ucp1*. The PCR products were electrophoresed in agarose gels, followed by staining with ethidium bromide. A representative result is shown.

Fig. 2. Immunolocalization of Ucp1 in subcutaneous WAT depot of fattening cattle

The subcutaneous WAT from cattle 31 months of age was fixed and sectioned, followed by immunochemical analyses to determine localization of Ucp1. Bar: 100 μm.

Fig. 3. Diet-related changes in Ucp1 expression in WAT depots of fattening cattle

Fattening cattle were fed either the roughage diet or the concentrate diet for 20 months. At 30 months of age, subcutaneous (sc), mesenteric (mesen), perirenal (pr), intermuscular (inter) and intramuscular (intra) WAT depots were collected, and *Ucp1* expression was examined by qRT-PCR. Expression of *Ucp1* was normalized to that of *Hprt1*, and the expression in the roughage diet group in each WAT depot was set to 1. Data are shown as the mean ± SE (n = 4). *: $P < 0.05$.

Fig. 4. Diet-related changes in expression of brown adipocyte-selective genes in WAT depots of fattening cattle

Fattening cattle were fed either the roughage diet or the concentrate diet for 20 months. Expression levels of brown adipocyte-selective genes in the subcutaneous WAT and the mesenteric WAT were examined by qRT-PCR. The expression was normalized to

515 *Hprt1* expression, and the expression in the roughage diet group in each WAT depot
516 was set to 1. Data are shown as the mean ± SE (n = 4). * and **: $P < 0.05$ and $P < 0.01$,
517 respectively.
518
519 Fig. 5. Diet-related expression of Prdm16 and leptin in WAT depots of fattening
520 cattle
521 Fattening cattle were fed either the roughage diet or the concentrate diet for 20 months.
522 At 30 months of age, subcutaneous (sc), mesenteric (mesen), perirenal (pr),
523 intermuscular (inter) and intramuscular (intra) WAT depots were collected, and
524 expression of *Prdm16* (A) and *leptin* (B) was examined by qRT-PCR. The expression
525 was normalized to *Hprt1* expression, and the expression in the roughage diet group in
526 each WAT depot was set to 1. Data are shown as the mean ± SE (n = 4). * and **: $P <$
527 0.05 and $P < 0.01$, respectively.
528

Table 1. Oligonucleotide PCR primers for conventional RT-PCR and quantitative RT-PCR

	Oligonucleotide		GenBank
	5′-primer	3′-primer	accession number
Conventional RT-PCR			
Ucp1	5’-agggactactcccaatctgaca-3’	5’-gttgggcacacttgtgtactgt-3’	XM_003587124
Quantitative RT-PCR			
Cidea	5’-agcaagaccttggatgcact-3’	5’-gaactcctctgtgtccaccac-3’	NM_001083449
Cox1	5’-gtttctgagtcgtcgcttcc-3’	5’-gggcaaagaaggcaaacat-3’	NM_001105323
Cox7a1	5’-cgagaaccgagtagctgagaa-3’	5’-atacaggatgttgtctgttgcac-3’	NM_176674
Cox8b	5’-cctaaggcacacatcactgc-3’	5’-aacgtcacagagagcccaat-3’	NM_001114517
Dio2	5’-atgccaccttctggactttg-3’	5’-ggcagctggttagtgaaagg-3’	NM_001010992
Hprt1	5’-gtgattagcgatgatgaaccag-3’	5’-ccatgaggaataaacaccttctc-3’	NM_001034035
Leptin	5’-acatctcacacacgcagtcc-3’	5’-ggatgaagtccaaaccagtga-3’	NM_173928
Pgc1α	5′-acctccatttttgagcatcag-3′	5′-acgcgccaaactttactgac-3′	NM_177945
Prdm16	5’-gagggctgcatccaaaag-3’	5’-agcatccacacagagcttcc-3’	XM_001788152
Ucp1	5′-ctgcgtggctgacataatca-3′	5′-tggatctgtagccggacttt-3′	XM_003587124